METHODS USING MECHANISMS OF ACTION OF AROA

FIELD OF THE INVENTION

This invention relates to newly identified polynucleotides and polypeptides, and their production and uses, as well as their variants, agonists and antagonists, and their uses. In particular, in these and in other regards, the invention relates to novel polynucleotides and polypeptides of the aro (5-enolpyruvylshikimate-3-phosphate synthase) family, hereinafter referred to as "aroA".

BACKGROUND OF THE INVENTION

The Streptococci make up a medically important genera of microbes known to cause several types of disease in humans, including, for example, otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid. Since its isolation more than 100 years ago, Streptococcus pneumoniae has been one of the more intensively studied microbes. For example, much of our early understanding that DNA is, in fact, the genetic material was predicated on the work of Griffith and of Avery, Macleod and McCarty using this microbe. Despite the vast amount of research *with S. pneumoniae*, many questions concerning the virulence of this microbe remain. It is particularly preferred to employ Streptococcal genes and gene products as targets for the development of antibiotics.

The frequency of *Streptococcus pneumoniae* infections has risen dramatically in the past 20 years. This has been attributed to the emergence of multiply antibiotic resistant strains and an increasing population of people with weakened immune systems. It is no longer uncommon to isolate *Streptococcus pneumoniae* strains which are resistant to some or all of the standard antibiotics. This has created a demand for both new anti-microbial agents and diagnostic tests for this organism.

Clearly, there is a need for factors, such as the novel compounds of the invention, that have a present benefit of being useful to screen compounds for antibiotic activity. Such factors are also useful to determine their role in pathogenesis of infection, dysfunction and disease. There is also a need for identification and characterization of such factors and their antagonists and agonists which can play a role in preventing, ameliorating or correcting infections, dysfunctions or diseases.

The polypeptides of the invention have amino acid sequence homology to a known *Lactococcus lactis* 5-enolpyruvoylshikimate -3-phosphate synthase (aro A) protein.

SUMMARY OF THE INVENTION

It is an object of the invention to provide polypeptides that have been identified as novel aroA polypeptides by homology between the amino acid sequence set out in Table 1 [SEQ ID NO: 2] and a known amino acid sequence or sequences of other proteins such as Lactococcus lactis 5-enolpyruvoylshikimate -3-phosphate synthase (aro A) protein.

It is a further object of the invention to provide polynucleotides that encode aroA polypeptides, particularly polynucleotides that encode the polypeptide herein designated aroA.

In a particularly preferred embodiment of the invention the polynucleotide comprises a region encoding aroA polypeptides comprising the sequence set out in Table 1 [SEQ ID NO:1] which includes a full length gene, or a variant thereof.

In another particularly preferred embodiment of the invention there is a novel aroA protein from *Streptococcus pneumoniae* comprising the amino acid sequence of Table 1 [SEQ ID NO:2], or a variant thereof.

In accordance with another aspect of the invention there is provided an isolated nucleic acid molecule encoding a mature polypeptide expressible by the *Streptococcus pneumoniae* 0100993 strain contained in the deposited strain.

A further aspect of the invention there are provided isolated nucleic acid molecules encoding aroA, particularly *Streptococcus pneumoniae* aroA, including mRNAs, cDNAs, genomic DNAs. Further embodiments of the invention include biologically, diagnostically, prophylactically, clinically or therapeutically useful variants thereof, and compositions comprising the same.

In accordance with another aspect of the invention, there is provided the use of a polynucleotide of the invention for therapeutic or prophylactic purposes, in particular genetic immunization. Among the particularly preferred embodiments of the invention are naturally occurring allelic variants of aroA and polypeptides encoded thereby.

Another aspect of the invention there are provided novel polypeptides of *Streptococcus pneumoniae* referred to herein as aroA as well as biologically, diagnostically, prophylactically, clinically or therapeutically useful variants thereof, and compositions comprising the same.

Among the particularly preferred embodiments of the invention are variants of aroA polypeptide encoded by naturally occurring alleles of the aroA gene.

In a preferred embodiment of the invention there are provided methods for producing the aforementioned aroA polypeptides.

In accordance with yet another aspect of the invention, there are provided inhibitors to such polypeptides, useful as antibacterial agents, including, for example, antibodies.

In accordance with certain preferred embodiments of the invention, there are provided products, compositions and methods for assessing aroA expression, treating disease, for example, otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid, assaying genetic variation, and administering a aroA polypeptide or polynucleotide to an organism to raise an immunological response against a bacteria, especially a *Streptococcus pneumoniae* bacteria.

In accordance with certain preferred embodiments of this and other aspects of the invention there are provided polynucleotides that hybridize to aroA polynucleotide sequences, particularly under stringent conditions.

In certain preferred embodiments of the invention there are provided antibodies against aroA polypeptides.

In other embodiments of the invention there are provided methods for identifying compounds which bind to or otherwise interact with and inhibit or activate an activity of a polypeptide or polynucleotide of the invention comprising: contacting a polypeptide or polynucleotide of the invention with a compound to be screened under conditions to permit binding to or other interaction between the compound and the polypeptide or polynucleotide to assess the binding to or other interaction with the compound, such binding or interaction being associated with a second component capable of providing a detectable signal in response to the binding or interaction of the polypeptide or polynucleotide with the compound; and determining whether the compound binds to or otherwise interacts with and activates or inhibits an activity of the polypeptide or polynucleotide by detecting the presence or absence of a signal generated from the binding or interaction of the compound with the polypeptide or polynucleotide.

In accordance with yet another aspect of the invention, there are provided aroA agonists and antagonists, preferably bacteriostatic or bacteriocidal agonists and antagonists.

In a further aspect of the invention there are provided compositions comprising a aroA polynucleotide or a aroA polypeptide for administration to a cell or to a multicellular organism.

The invention provides an antagonist that inhibits or an agonist that activates an activity a polypeptide selected from the group consisting of: a polypeptide comprising an amino acid sequence which is at least 90% identical to the amino acid sequence of SEQ ID NO:2 or 4, and a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2 or 4.

In any of the methods herein using or referring to an activity of AroA or a polypeptide of the invention, said activity is selected from the group consisting of: synthesis of p-aminobenzoate, synthesis of ubiquinone, transformation of phospho(enol)pyruvate (PEP) and shikimate 3-phosphate (S3P) to EPSP and inorganic phosphate (Pi), transformation of EPSP and inorganic phosphate (Pi) to phospho(enol)pyruvate (PEP) and shikimate 3-phosphate (S3P), binding of AroA and phospho(enol)pyruvate, binding of AroA to phospho(enol)pyruvate-pyruvate kinase complex, binding of AroA and phospho(enol)pyruvate-lactacte dehydrogenase complex, binding of AroA and shikimate 3-phosphate, competitive inhibition of the forward reaction of AroA by glyphosate (herein also "GLP") versus phospho(enol)pyruvate,uncompetitive inhibition of the forward reaction of AroA by glyphosate versus shikimate 3-phosphate, competitive inhibition of the forward reaction of AroA by EPSP versus phospho(enol)pyruvate, competitive inhibition of the forward reaction of AroA by EPSP versus shikimate 3-phosphate, uncompetitive inhibition of the reverse reaction of AroA by glyphosate versus EPSP, noncompetitive inhibition of the reverse reaction of AroA by glyphosate versus Pi, competitive inhibition of the reverse reaction of AroA by shikimate 3-phosphate versus EPSP, and uncompetitive inhibition of the reverse reaction of AroA by shikimate 3-phosphate versus Pi.

Further provided by the invention is a method for the treatment of an individual having need to inhibit or activate AroA polypeptide comprising the steps of: administering to the individual a antibacterially effective amount of an antagonist that inhibits or an agonist that activates an activity of a polypeptide selected from the group consisting of: a polypeptide comprising an amino acid sequence which is at least 90% identical to the amino acid sequence of SEQ ID NO:2 or 4, and a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2 or 4

Also provided is a method for the treatment of an individual infected with a bacteria comprising the steps of administering to the individual a antibacterially effective amount of an

antagonist that inhibits or an agonist that activates an activity of a polypeptide selected from the group consisting of: a polypeptide comprising an amino acid sequence which is at least 90% identical to the amino acid sequence of SEQ ID NO:2 or 4, and a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2 or 4.

In any of the methods of the ivention, it is preferred that a bacteria is selected from the group consisting of a member of the genus *Staphylococcus*, *Staphylococcus aureus*, a member of the genus *Streptococcus*, and *Streptococcus pneumoniae*.

Also provided by invention is a method for the treatment of an individual having need to inhibit or activate AroA polypeptide comprising the steps of administering to the individual a antibacterially effective amount of an antagonist that inhibits or an agoinist that activates an activity of AroA.

The invention also provides a method for the treatment of an individual infected with a bacteria comprising the steps of administering to the individual a antibacterially effective amount of an antagonist that inhibits or an agonist that activates that activates an activity of AroA.

A method is also provided for the treatment of an individual infected by a bacteria comprising the steps of administering to the individual an antibacterially effective amount of a compound that is a competitive inhibitor of shikimate-3-phosphate substrate utilization by AroA. In a preferred embodiment a compound of the invention, such as an antagonist or agonist, binds to or interacts with the active site of AroA or interacts with shikimate-3-phosphate or interferes with the binding of shikimate-3-phosphate.

A method for the treatment of an individual infected by *Streptococcus pneumoniae* comprising the steps of administering to the individual a antibacterially effective amount of an antagonist that inhibits or anagonist that activates an activity of *Streptococcus pneumoniae* AroA is also provided by the invention.

Further provided is an antagonist that inhibits an activity of a polypeptide selected from the group consisting of: a polypeptide comprising an amino acid sequence which is at least 90% identical to the amino acid sequence of SEQ ID NO:2 or 4, and a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2 or 4.

A method is further provided by the invention for the treatment of an individual having need to inhibit AroA polypeptide comprising the steps of administering to the individual a antibacterially effective amount of an antagonist that inhibits an activity of a polypeptide selected from the group consisting of: a polypeptide comprising an amino acid

sequence which is at least 90% identical to the amino acid sequence of SEQ ID NO:2 or 4, and a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2 or 4.

Still further provided is a method for inhibiting an activity of AroA polypeptide comprising the steps of contacting a composition comprising said polypeptide with an effective amount of an antagonist that inhibits an activbity of AroA.

Another embodiment of the invention is a method for inhibiting a growth of bacteria comprising the steps of contacting a composition comprising bacteria with an antibacterially effective amount of an antagonist that inhibits an activity of AroA.

A still further embodiment of the invention is a a method for inhibiting a AroA polypeptide comprising the steps of contacting a composition comprising bacteria with an antibacterially effective amount of an antagonist that inhibits an activity of AroA.

Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following descriptions and from reading the other parts of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Inhibition of EPSP synthase by GLP in the forward reaction. Panel A: Competitive inhibition versus PEP determined at fixed S3P (1 mM); [GLP] = 0 (○), 0.5 ⁻ M (●), 3 microMolar (□), and 6 microMolar (■). Panel B: Uncompetitive inhibition versus S3P determined at fixed PEP (1 mM); [GLP] = 0 (○), 25 microMolar (●), 50 microMolar (□), and 100 microMolar (■).

Figure 2. Inhibition of EPSP synthase by EPSP in the forward reaction. Panel A: Competitive inhibition versus PEP determined at fixed S3P (1 mM); [EPSP] = 0 (○), 50 microMolar (●), 100 microMolar (□), 200 microMolar (■), and 400 microMolar ( ). Panel B: Competitive inhibition versus S3P determined at fixed PEP (1 mM); [EPSP] = 0 (○), 25 microMolar (●), 50 microMolar (□), 75 microMolar (■), and 100 microMolar ( ).

Figure 3. Inhibition of EPSP synthase by GLP in the reverse reaction. Panel A: Mixed inhibition versus EPSP determined at fixed Pi (10 mM); [GLP] = 0 (○), 0.25 mM (●), 0.5 mM (□), 0.75 mM (■), and 1 mM ( ). Panel B: Mixed inhibition versus Pi determined at fixed EPSP (0.5 mM); [GLP] = 0 (○), 0.1 mM (●), 0.5 mM (□), and 1 mM (■).

Figure 4. Inhibition of EPSP synthase by S3P in the reverse reaction. Panel A: Competitive inhibition versus EPSP at fixed Pi (10 mM); [S3P] = 0 (○), 0.1 mM (●), 0.25 mM (□), and 0.5 mM (■). Panel B: Uncompetitive inhibition versus Pi at fixed EPSP (0.5 mM); [S3P] = 0 (○), 0.1 mM (●), 0.25 mM (□), and 0.5 mM (■).

Figure 5 (Schemes 1 and 2) shows schemes for the EPSP synthase-catalyzed reaction and the formation of the PEP oxonium ion.

Figure 6 (Scheme 3) shows a proposed mechanism for the forward reaction of *S. pneumoniae* EPSP synthase.

GLOSSARY

The following definitions are provided to facilitate understanding of certain terms used frequently herein.

"Bodily material(s) means any material derived from an individual or from an organism infecting, infesting or inhabiting an individual, including but not limited to, cells, tissues and waste, such as, bone, blood, serum, cerebrospinal fluid, semen, saliva, muscle, cartilage, organ tissue, skin, urine, stool or autopsy materials.

"Disease(s)" means any disease caused by or related to infection by a bacteria, including , for example, infections of the upper respiratory tract (e.g., otitis media, bacterial tracheitis, acute epiglottitis, thyroiditis), lower respiratory (e.g., empyema, lung abscess), cardiac (e.g., infective endocarditis), gastrointestinal (e.g., secretory diarrhoea, splenic absces, retroperitoneal abscess), CNS (e.g., cerebral abscess), eye (e.g., blepharitis, conjunctivitis, keratitis, endophthalmitis, preseptal and orbital cellulitis, darcryocystitis), kidney and urinary tract (e.g., epididymitis, intrarenal and perinephric absces, toxic shock syndrome), skin (e.g., impetigo, folliculitis, cutaneous abscesses, cellulitis, wound infection, bacterial myositis) bone and joint (e.g., septic arthritis, osteomyelitis).

"Host cell(s)" is a cell that has been introduced (*e.g.*, transformed or transfected) or is capable of introduction (*e.g.*, transformation or transfection) by an exogenous polynucleotide sequence.

"Identity," as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" can be readily calculated by known methods, including but not limited to those described in (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and

Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM *J. Applied Math.*, *48:* 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., *Nucleic Acids Research 12(1):* 387 (1984)), BLASTP, BLASTN, and FASTA (Altschul, S.F. et al., *J. Molec. Biol. 215:* 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al.*, *J. Mol. Biol. 215*: 403-410 (1990). The well known Smith Waterman algorithm may also be used to determine identity.

Parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992)

Gap Penalty: 12

Gap Length Penalty: 4

A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

Parameters for polynucleotide comparison include the following: Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970)

Comparison matrix: matches = +10, mismatch = 0

Gap Penalty: 50

Gap Length Penalty: 3

Available as: The "gap" program from Genetics Computer Group, Madison WI. These are the default parameters for nucleic acid comparisons.

A preferred meaning for "identity" for polynucleotides and polypeptides, as the case may be, are provided in (1) and (2) below.

(1) Polynucleotide embodiments further include an isolated polynucleotide comprising a polynucleotide sequence having at least a 95, 97 or 100% identity to the reference sequence of SEQ ID NO:1 OR 3, wherein said polynucleotide sequence may be identical to the reference sequence of SEQ ID NO:1 OR 3 or may include up to a certain integer number of nucleotide alterations as compared to the reference sequence, wherein said

alterations are selected from the group consisting of at least one nucleotide deletion, substitution, including transition and transversion, or insertion, and wherein said alterations may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among the nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of nucleotide alterations is determined by multiplying the total number of nucleotides in SEQ ID NO:1 OR 3 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of nucleotides in SEQ ID NO:1 OR 3, or:

$$n_n \leq x_n - (x_n \bullet y),$$

wherein n_n is the number of nucleotide alterations, x_n is the total number of nucleotides in SEQ ID NO:1 OR 3, y is 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and $\bullet$ is the symbol for the multiplication operator, and wherein any non-integer product of x_n and y is rounded down to the nearest integer prior to subtracting it from x_n. Alterations of a polynucleotide sequence encoding the polypeptide of SEQ ID NO:2 OR 4 may create nonsense, missense or frameshift mutations in this coding sequence and thereby alter the polypeptide encoded by the polynucleotide following such alterations.

(2) Polypeptide embodiments further include an isolated polypeptide comprising a polypeptide having at least a 95, 97 or 100% identity to a polypeptide reference sequence of SEQ ID NO:2 OR 4, wherein said polypeptide sequence may be identical to the reference sequence of SEQ ID NO:2 OR 4 or may include up to a certain integer number of amino acid alterations as compared to the reference sequence, wherein said alterations are selected from the group consisting of at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence, and wherein said number of amino acid alterations is determined by multiplying the total number of amino acids in SEQ ID NO:2 OR 4 by the integer defining the percent identity divided by 100 and then subtracting that product from said total number of amino acids in SEQ ID NO:2 OR 4, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein n_a is the number of amino acid alterations, x_a is the total number of amino acids in SEQ ID NO:2 OR 4, y is 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and • is the symbol for the multiplication operator, and wherein any non-integer product of x_a and y is rounded down to the nearest integer prior to subtracting it from x_a.

"Individual(s)" means a multicellular eukaryote, including, but not limited to a metazoan, a mammal, an ovid, a bovid, a simian, a primate, and a human.

"Isolated" means altered "by the hand of man" from its natural state, *i.e.*, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or a polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Moreover, a polynucleotide or polypeptide that is introduced into an organism by transformation, genetic manipulation or by any other recombinant method is "isolated" even if it is still present in said organism, which organism may be living or non-living.

"Organism(s)" means a (i) prokaryote, including but not limited to, a member of the genus *Streptococcus, Staphylococcus, Bordetella, Corynebacterium, Mycobacterium, Neisseria, Haemophilus, Actinomycetes, Streptomycetes, Nocardia, Enterobacter, Yersinia, Fancisella, Pasturella, Moraxella, Acinetobacter, Erysipelothrix, Branhamella, Actinobacillus, Streptobacillus, Listeria, Calymmatobacterium, Brucella, Bacillus, Clostridium, Treponema, Escherichia, Salmonella, Kleibsiella, Vibrio, Proteus, Erwinia, Borrelia, Leptospira, Spirillum, Campylobacter, Shigella, Legionella, Pseudomonas, Aeromonas, Rickettsia, Chlamydia, Borrelia* and *Mycoplasma*, and further including, but not limited to, a member of the species or group, Group A *Streptococcus*, Group B *Streptococcus*, Group C *Streptococcus*, Group D *Streptococcus*, Group G *Streptococcus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus faecalis*, *Streptococcus faecium*, *Streptococcus durans*, *Neisseria gonorrheae*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Corynebacterium diptheriae*, *Gardnerella vaginalis*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium ulcerans*, *Mycobacterium leprae*, *Actinomyctes israelii*, *Listeria monocytogenes*, *Bordetella pertusis*, *Bordatella parapertusis*, *Bordetella bronchiseptica*, *Escherichia coli*, *Shigella dysenteriae*, *Haemophilus influenzae*, *Haemophilus*

aegyptius, Haemophilus parainfluenzae, Haemophilus ducreyi, Bordetella, Salmonella typhi, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris, Yersinia pestis, Kleibsiella pneumoniae, Serratia marcessens, Serratia liquefaciens, Vibrio cholera, Shigella dysenterii, Shigella flexneri, Pseudomonas aeruginosa, Franscisella tularensis, Brucella abortis, Bacillus anthracis, Bacillus cereus, Clostridium perfringens, Clostridium tetani, Clostridium botulinum, Treponema pallidum, Rickettsia rickettsii and *Chlamydia trachomitis*, (ii) an archaeon, including but not limited to *Archaebacter*, and (iii) a unicellular or filamentous eukaryote, including but not limited to, a protozoan, a fungus, a member of the genus *Saccharomyces, Kluveromyces*, or *Candida*, and a member of the species *Saccharomyces ceriviseae, Kluveromyces lactis*, or *Candida albicans*.

"Bacteria(um)" means a (i) prokaryote, including but not limited to, a member of the genus *Streptococcus, Staphylococcus, Bordetella, Corynebacterium, Mycobacterium, Neisseria, Haemophilus, Actinomycetes, Streptomycetes, Nocardia, Enterobacter, Yersinia, Fancisella, Pasturella, Moraxella, Acinetobacter, Erysipelothrix, Branhamella, Actinobacillus, Streptobacillus, Listeria, Calymmatobacterium, Brucella, Bacillus, Clostridium, Treponema, Escherichia, Salmonella, Kleibsiella, Vibrio, Proteus, Erwinia, Borrelia, Leptospira, Spirillum, Campylobacter, Shigella, Legionella, Pseudomonas, Aeromonas, Rickettsia, Chlamydia, Borrelia* and *Mycoplasma*, and further including, but not limited to, a member of the species or group, Group A *Streptococcus*, Group B *Streptococcus*, Group C *Streptococcus*, Group D *Streptococcus*, Group G *Streptococcus, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus faecalis, Streptococcus faecium, Streptococcus durans, Neisseria gonorrheae, Neisseria meningitidis, Staphylococcus aureus, Staphylococcus epidermidis, Corynebacterium diptheriae, Gardnerella vaginalis, Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium ulcerans, Mycobacterium leprae, Actinomyctes israelii, Listeria monocytogenes, Bordetella pertusis, Bordatella parapertusis, Bordetella bronchiseptica, Escherichia coli, Shigella dysenteriae, Haemophilus influenzae, Haemophilus aegyptius, Haemophilus parainfluenzae, Haemophilus ducreyi, Bordetella, Salmonella typhi, Citrobacter freundii, Proteus mirabilis, Proteus vulgaris, Yersinia pestis, Kleibsiella pneumoniae, Serratia marcessens, Serratia liquefaciens, Vibrio cholera, Shigella dysenterii, Shigella flexneri, Pseudomonas aeruginosa, Franscisella tularensis, Brucella abortis, Bacillus anthracis, Bacillus cereus, Clostridium perfringens, Clostridium tetani, Clostridium botulinum, Treponema pallidum, Rickettsia rickettsii* and *Chlamydia trachomitis*, and (ii) an archaeon, including but not limited to *Archaebacter*.

"Polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxyribonucleotide, that may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotide(s)" include, without limitation, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded regions, or a mixture of single- and double-stranded regions. In addition, "polynucleotide" as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term "polynucleotide(s)" also includes DNAs or RNAs as described above that comprise one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotide(s)" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term "polynucleotide(s)" as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including, for example, simple and complex cells. "Polynucleotide(s)" also embraces short polynucleotides often referred to as oligonucleotide(s).

"Polypeptide(s)" refers to any peptide or protein comprising two or more amino acids joined to each other by peptide bonds or modified peptide bonds. "Polypeptide(s)" refers to both short chains, commonly referred to as peptides, oligopeptides and oligomers and to longer chains generally referred to as proteins. Polypeptides may comprise amino acids other than the 20 gene encoded amino acids. "Polypeptide(s)" include those modified either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may comprise many types of modifications. Modifications can occur anywhere in a

polypeptide, including the peptide backbone, the amino acid side-chains, and the amino or carboxyl termini. Modifications include, for example, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphotidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cysteine, formation of pyroglutamate, formylation, gamma-carboxylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins, such as arginylation, and ubiquitination. See, for instance, *PROTEINS - STRUCTURE AND MOLECULAR PROPERTIES*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993) and Wold, F., Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., *Meth. Enzymol. 182*:626-646 (1990) and Rattan et al., *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N.Y. Acad. Sci. 663: 48-62 (1992). Polypeptides may be branched or cyclic, with or without branching. Cyclic, branched and branched circular polypeptides may result from post-translational natural processes and may be made by entirely synthetic methods, as well.

"Recombinant expression system(s)" refers to expression systems or portions thereof or polynucleotides of the invention introduced or transformed into a host cell or host cell lysate for the production of the polynucleotides and polypeptides of the invention.

"Variant(s)" as the term is used herein, is a polynucleotide or polypeptide that differs from a reference polynucleotide or polypeptide respectively, but retains essential properties. A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, additions, deletions, fusion proteins and truncations in the polypeptide encoded by the reference sequence, as discussed below. A typical variant of a polypeptide differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more

substitutions, additions, deletions in any combination. A substituted or inserted amino acid residue may or may not be one encoded by the genetic code. The present invention also includes include variants of each of the polypeptides of the invention, that is polypeptides that vary from the referents by conservative amino acid substitutions, whereby a residue is substituted by another with like characteristics. Typical such substitutions are among Ala, Val, Leu and Ile; among Ser and Thr; among the acidic residues Asp and Glu; among Asn and Gln; and among the basic residues Lys and Arg; or aromatic residues Phe and Tyr. Particularly preferred are variants in which several, 5-10, 1-5, 1-3, 1-2 or 1 amino acids are substituted, deleted, or added in any combination. A variant of a polynucleotide or polypeptide may be a naturally occurring such as an allelic variant, or it may be a variant that is not known to occur naturally. Non-naturally occurring variants of polynucleotides and polypeptides may be made by mutagenesis techniques, by direct synthesis, and by other recombinant methods known to skilled artisans.

DESCRIPTION OF THE INVENTION

The invention relates to novel aroA polypeptides and polynucleotides as described in greater detail below. In particular, the invention relates to polypeptides and polynucleotides of a novel aroA of *Streptococcus pneumoniae*, which is related by amino acid sequence homology to Lactococcus lactis 5-enolpyruvoylshikimate -3-phosphate synthase (aro A) Percent Similarity: 79.673, Percent Identity: 68.224 polypeptide. The invention relates especially to aroA having the nucleotide and amino acid sequences set out in Table 1 [SEQ ID NO: 1] and Table 1 [SEQ ID NO: 2] respectively, and to the aroA nucleotide sequences of the DNA in the deposited strain and amino acid sequences encoded thereby.

TABLE 1

aroA Polynucleotide and Polypeptide Sequences

(A) Sequences from *Streptococcus pneumoniae* aroA polynucleotide sequence [SEQ ID NO:1].

5'-ATGAAACTAA AAACAAACAT TCGCCATTTA CATGGTATTA TCCGCGTCCCAGGTGACAAG
TCTATCAGCC ACCGTTCCAT TATCTTTGGA AGTTTGGCTG AGGGTGAGAC CAAGGTTTAT
GATATTCTGC GAGGTGAAGA CGTTCTTTCGACCATGCAGG TTTTTCGTGA CCTTGGTGTT
GAAATTGAGG ATAAAGATGG GGTTATTACC GTTCAAGGTG TAGGCATGGC TGGCTTAAAA
GCGCCGCAAA ATGCCCTTAA TATGGGAAAT TCTGGCACCT CGATTCGCCT GATTTCAGGT

GTCCTTGCTG GTGCAGATTT CGAAGTAGAG ATGTTTGGAG ATGATAGTCT TTCCAAACGT
CCTATGGACC GTGTGACCCT TCCACTGAAA AAAATGGGCG TCAGCATCTC AGGGCAAACT
GAACGAGACT TGCCTCCCCT TCGCTTAAAA
GGGACGAAAA ACCTAAGACC TATTCATTAT GAGTTGCCAA TTGCCTCTGC CCAAGTCAAG
TCAGCCTTGA TGTTTGCAGC CTTACAAGCT AAGGGGGAGTCAGTTATTAT CGAAAAAGAG
TACACCCGTA ATCATACTGA AGATATGTTG CAACAATTTG GTGGTCATTT AAGTGTGGAT
GGTAAGAAAA TCACAGTCCA AGGCCCACAA AAATTGACAG GACAGAAGGT GGTCGTACCA
GGAGATATTT CCAGTGCAGC CTTTTGGTTA GTCGCAGGTT TGATTGCTCC AAATTCTCGT
CTAGTGCTGC AGAATGTGGG GATAAACGAA ACTCGCACCG GTATTATTGATGTCATTCGT
GCCATGGGTG GAAAATTGGA AATAACTGAA ATCGATCCAG TCGCTAAATC TGCAACCTTG
ATTGTTGAGT CTTCTGACTT GAAAGGAACA GAGATTTGTG GCGCTTTGAT TCCACGTTTG
ATTGATGAAT TGCCTATTAT TGCCCTACTT GCGACCCAAG CCCAAGGTGT AACAGTTATC
AAGGATGCTG AGGAGCTCAA GGTCAAGGAA ACAGACCGTA TTCAGGTTGT GGCAGACGCC
TTAAATAGTA TGGGAGCAGA TATTACTCCT ACGGCAGATG GGATGATTAT CAAAGGAAAA
TCAGCTCTTC ACGGTGCTAG AGTCAATACG TTTGGTGACC ACCGTATCGG CATGATGACA
GCTATCGCAG CCCTATTGGT TGCAGATGGAGAGGTGGAGC TTGACCGTGC AGAAGCCATC
AATACCAGCT ATCCTAGTTT CTTTGATGAT TTGGAGAGCT TGATTCATGG CTAA-3'

(B) aroA polypeptide sequence deduced from the polynucleotide sequence in this table [SEQ ID NO:2].

NH_2-MKLKTNIRHL HGIIRVPGDK SISHRSIIFG SLAEGETKVY DILRGEDVLSTMQVFRDLGV
EIEDKDGVIT VQGVGMAGLK APQNALNMGN SGTSIRLISGVLAGADFEVE MFGDDSLSKR
PMDRVTLPLK KMGVSISGQT ERDLPPLRLK GTKNLRPIHY ELPIASAQVK SALMFAALQA
KGESVIIEKE YTRNHTEDMLQQFGGHLSVD GKKITVQGPQ KLTGQKVVVP GDISSAAFWL
VAGLIAPNSRLVLQNVGINE TRTGIIDVIR AMGGKLEITE IDPVAKSATL IVESSDLKGT
EICGALIPRL IDELPIIALL ATQAQGVTVI KDAEELKVKE TDRIQVVADA LNSMGADITP
TADGMIIKGK SALHGARVNT FGDHRIGMMT AIAALLVADGEVELDRAEAI NTSYPSFFDD
LESLIHG-COOH

(C) Polynucleotide sequence embodiments [SEQ ID NO:1].

X-$(R_1)_n$-ATGAAACTAA AAACAAACAT TCGCCATTTA CATGGTATTA
TCCGCGTCCCAGGTGACAAG TCTATCAGCC ACCGTTCCAT TATCTTTGGA AGTTTGGCTG
AGGGTGAGAC CAAGGTTTAT GATATTCTGC GAGGTGAAGA CGTTCTTTCGACCATGCAGG
TTTTTCGTGA CCTTGGTGTT GAAATTGAGG ATAAAGATGG GGTTATTACC GTTCAAGGTG
TAGGCATGGC TGGCTTAAAA GCGCCGCAAA ATGCCCTTAA TATGGGAAAT TCTGGCACCT
CGATTCGCCT GATTTCAGGT GTCCTTGCTG GTGCAGATTT CGAAGTAGAG ATGTTTGGAG
ATGATAGTCT TTCCAAACGT CCTATGGACC GTGTGACCCT TCCACTGAAA AAAATGGGCG
TCAGCATCTC AGGGCAAACT GAACGAGACT TGCCTCCCCT TCGCTTAAAA
GGGACGAAAA ACCTAAGACC TATTCATTAT GAGTTGCCAA TTGCCTCTGC CCAAGTCAAG
TCAGCCTTGA TGTTTGCAGC CTTACAAGCT AAGGGGGAGTCAGTTATTAT CGAAAAAGAG
TACACCCGTA ATCATACTGA AGATATGTTG CAACAATTTG GTGGTCATTT AAGTGTGGAT
GGTAAGAAAA TCACAGTCCA ACGGCCACAA AAATTGACAG GACAGAAGGT GGTCGTACCA
GGAGATATTT CCAGTGCAGC CTTTTGGTTA GTCGCAGGTT TGATTGCTCC AAATTCTCGT

CTAGTGCTGC AGAATGTGGG GATAAACGAA ACTCGCACCG GTATTATTGATGTCATTCGT
GCCATGGGTG GAAAATTGGA AATAACTGAA ATCGATCCAG TCGCTAAATC TGCAACCTTG
ATTGTTGAGT CTTCTGACTT GAAAGGAACA GAGATTTGTG GCGCTTTGAT T
CCACGTTTG ATTGATGAAT TGCCTATTAT TGCCCTACTT GCGACCCAAG CCCAAGGTGT
AACAGTTATC AAGGATGCTG AGGAGCTCAA GGTCAAGGAA ACAGACCGTA TTCAGGTTGT
GGCAGACGCC TTAAATAGTA TGGGAGCAGA TATTACTCCT ACGGCAGATG GGATGATTAT
CAAAGGAAAA TCAGCTCTTC ACGGTGCTAG AGTCAATACG TTTGGTGACC ACCGTATCGG
CATGATGACA GCTATCGCAG CCCTATTGGT TGCAGATGGAGAGGTGGAGC TTGACCGTGC
AGAAGCCATC AATACCAGCT ATCCTAGTTT CTTTGATGAT TTGGAGAGCT TGATTCATGG
CTAA-$(R_2)_n$-Y

(D) Polypeptide sequence embodiments [SEQ ID NO:2].

X-$(R_1)_n$-MKLKTNIRHL HGIIRVPGDK SISHRSIIFG SLAEGETKVY
DILRGEDVLSTMQVFRDLGV EIEDKDGVIT VQGVGMAGLK APQNALNMGN
SGTSIRLISGVLAGADFEVE MFGDDSLSKR PMDRVTLPLK KMGVSISGQT ERDLPPLRLK
GTKNLRPIHY ELPIASAQVK SALMFAALQA KGESVIIEKE YTRNHTEDMLQQFGGHLSVD
GKKITVQGPQ KLTGQKVVVP GDISSAAFWL VAGLIAPNSR LVLQNVGINE TRTGIIDVIR
AMGGKLEITE IDPVAKSATL IVESSDLKGT EICGALIPRL IDELPIIALL ATQAQGVTVI
KDAEELKVKE TDRIQVVADA LNSMGADITP TADGMIIKGK SALHGARVNT FGDHRIGMMT
AIAALLVADGEVELDRAEAI NTSYPSFFDD LESLIHG
$-(R_2)_n$-Y

(E) Sequences from Streptococcus pneumoniae aroA polynucleotide ORF sequence [SEQ ID NO:3].

5'-AGCTTGATCG TCCCAGGTGA CAAGTCTATC AGCCACCGTT CCATTATCTT
TGGAAGTTTG GCTGAGGGTG AGACCAAGGT TTATGATATT CTGCGAGGTG AACACGTTCT
TTCGACCATG CAGGTTTTTC GTGACCTTGG TGTTGAAATT GAGGATAAAG ATGGGGTTAT
TACCGTTCAA GGTGTAGGCA TGGCTGGCTT AAAAGCGCCG CAAAATGCCC TTAATATGGG
AAATTCTGGC ACCTCGATTC
GCCTGATTTC AGGTGTCCTT GCTGGTGCAG ATTTCGAAGT AGAGATGTTT GGAGATGATA
GTCTTTCCAA ACGTCCTATG GACCGTGTGA CCCTTCCACT
GAAAAAAATG GGCGTCAGCA TCTCAGGGCA AACTGAACGA GACTTGCCTC
CCCTTCGCTT TAAAAGGGAC GAAAAACCTA AGACCTATtc attatgagtt gccaattgcc
tctgcccaag tcaagtcagc cnnnnnnnnn nnnnnnnnn CTAAGGGG GAGTCAGTTA
TTATCGAAAA AGAGTACACC CGTAATCATA CTGAAGATAT GTTGCAACAA TTTGGTGGTC
ATTTAAGTGT GGATGGTAAGAAAATCACAG TCCAAGGGCC ACAAAAATTG ACAGGACAGA
AGGTGGTCGT ACCAGGAGAT ATTTCCAGTG CAGCCTTTTG GTTAGTCGCA GGTTTGATTG
CTCCAAATTC TCGTCTAGTG CTGCAGAATG TGGGGATAAA CGAAACTCGC ACCGGTATTA
TTGATGTCAT TCGTGCCATG GGTGGAAAAT TGGAAATAACTGAAATCGAT CCAGTCGCTA
AATCTGCAAC CTTGATTGTT GAGTCTTCTGACTTGAAAGG AACAGAGATT TGTGGCGCTT
TGATTCCACG TTTGATTGAT
GAATTGCCTA TTATTGCCCT ACTTGCGACC CAAGCCCAAG GTGTAACAGT TATCAAGGAT
GCTGAGGAGC TCAAGGTCAA GGAAACAGAC CGTATTCAGG TTGTGGCAGA CGCCTTAAAT
AGTATGGGAG CAGATATTAC TCCTACGGCA GATGGGATGA TTATCAAAGG AAAATCAGCT
CTTCACGGTG CTAGAGTCAATACGTTTGGT GACCACCGTA TCGGCATGAT GACAGCTATC
GCAGCCCTAT TGGTTGCAGA TGGAGAGGTG GAGCTTGACC GTGCAGAAGC CATCAATACC
AGCTATCCTA GTTTCTTTGA TGATTTGGAG AGCTTGATTC ATGGCTAA
-3'

(F) aroA polypeptide sequence deduced from the polynucleotide ORF sequence in this table [SEQ ID NO:4].

NH$_2$-SLIVPGDKSI SHRSIIFGSL AEGETKVYDI LRGEHVLSTM QVFRDLGVEIEDKDGVITVQ
GVGMAGLKAP QNALNMGNSG TSIRLISGVL AGADFEVEMF GDDSLSKRPM DRVTLPLKKM
GVSISGQTER DLPPLRFKRD EKPKTYSLXV
ANCLCPSQVS XXXXXXXXKG ESVIIEKEYT RNHTEDMLQQ FGGHLSVDGK KITVQGPQKL
TGQKVVVPGD ISSAAFWLVA GLIAPNSRLV LQNVGINETR TGIIDVIRAM GGKLEITEID
PVAKSATLIV ESSDLKGTEI CGALIPRLID ELPIIALLAT QAQGVTVIKD AEELKVKETD
RIQVVADALN SMGADITPTA DGMIIKGKSA LHGARVNTFG DHRIGMMTAI AALLVADGEV
ELDRAEAINTSYPSFFDDLE SLIHG-COOH

Deposited materials

A deposit containing a *Streptococcus pneumoniae* 0100993 strain has been deposited with the National Collections of Industrial and Marine Bacteria Ltd. (herein "NCIMB"), 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland on 11 April 1996 and assigned deposit number 40794. The deposit was described as Streptococcus peumnoiae 0100993 on deposit. On 17 April 1996 a Streptococcus peumnoiae 0100993 DNA library in E. coli was similarly depositedwith the NCIMB and assigned deposit number 40800.. The *Streptococcus pneumoniae* strain deposit is referred to herein as "the deposited strain" or as "the DNA of the deposited strain."

The deposited strain contains the full length aroA gene. The sequence of the polynucleotides contained in the deposited strain, as well as the amino acid sequence of the polypeptide encoded thereby, are controlling in the event of any conflict with any description of sequences herein.

The deposit of the deposited strain has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for Purposes of Patent Procedure. The strain will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposited strain is provided merely as convenience to those of skill in the art and is not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112.

A license may be required to make, use or sell the deposited strain, and compounds derived therefrom, and no such license is hereby granted.

Polypeptides

The polypeptides of the invention include the polypeptide of Table 1 [SEQ ID NO:2] (in particular the mature polypeptide) as well as polypeptides and fragments, particularly those which have the biological activity of aroA, and also those which have at least 70% identity to a polypeptide of Table 1 [SEQ ID NOS:2 and 4] or the relevant portion, preferably at least

80% identity to a polypeptide of Table 1 [SEQ ID NOS:2 and 4], and more preferably at least 90% similarity (more preferably at least 90% identity) to a polypeptide of Table 1 [SEQ ID NOS:2 and 4] and still more preferably at least 95% similarity (still more preferably at least 95% identity) to a polypeptide of Table 1 [SEQ ID NOS:2 and 4] and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

The invention also includes polypeptides of the formula set forth in Table 1 (D) [SEQ ID NO:2] wherein, at the amino terminus, X is hydrogen, and at the carboxyl terminus, Y is hydrogen or a metal, R_1 and R_2 is any amino acid residue, and n is an integer between 1 and 1000. Any stretch of amino acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

A fragment is a variant polypeptide having an amino acid sequence that entirely is the same as part but not all of the amino acid sequence of the aforementioned polypeptides. As with aroA polypeptides fragments may be "free-standing," or comprised within a larger polypeptide of which they form a part or region, most preferably as a single continuous region, a single larger polypeptide.

Preferred fragments include, for example, truncation polypeptides having a portion of an amino acid sequence of Table 1 [SEQ ID NOS:2 and 4], or of variants thereof, such as a continuous series of residues that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus. Degradation forms of the polypeptides of the invention in a host cell, particularly a *Streptococcus pneumoniae*, are also preferred. Further preferred are fragments characterized by structural or functional attributes such as fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions.

Also preferred are biologically active fragments which are those fragments that mediate activities of aroA, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Also included are those fragments that are antigenic or immunogenic in an animal, especially in a human. Particularly preferred are fragments comprising receptors or domains of enzymes that confer a function essential for viability of *Streptococcus pneumoniae* or the ability to initiate, or maintain cause disease in an individual, particularly a human.

"X" or "Xaa" may also be used in describing certain polypeptides of the invention. "X" and "Xaa" mean that any of the twenty naturally occurring amino acids may appear at such a designated position in the polypeptide sequence.

Variants that are fragments of the polypeptides of the invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, these variants may be employed as intermediates for producing the full-length polypeptides of the invention.

Polynucleotides

Another aspect of the invention relates to isolated polynucleotides, including the full length gene, that encode the aroA polypeptide having a deduced amino acid sequence of Table 1 [SEQ ID NOS:2 and 4] and polynucleotides closely related thereto and variants thereof.

Using the information provided herein, such as a polynucleotide sequence set out in Table 1 [SEQ ID NOS:1 and 3], a polynucleotide of the invention encoding aroA polypeptide may be obtained using standard cloning and screening methods, such as those for cloning and sequencing chromosomal DNA fragments from bacteria using *Streptococcus pneumoniae* 0100993 cells as starting material, followed by obtaining a full length clone. For example, to obtain a polynucleotide sequence of the invention, such as a sequence given in Table 1 [SEQ ID NOS:1 and 3], typically a library of clones of chromosomal DNA of *Streptococcus pneumoniae* 0100993 in *E.coli* or some other suitable host is probed with a radiolabeled oligonucleotide, preferably a 17-mer or longer, derived from a partial sequence. Clones carrying DNA identical to that of the probe can then be distinguished using stringent conditions. By sequencing the individual clones thus identified with sequencing primers designed from the original sequence it is then possible to extend the sequence in both directions to determine the full gene sequence. Conveniently, such sequencing is performed using denatured double stranded DNA prepared from a plasmid clone. Suitable techniques are described by Maniatis, T., Fritsch, E.F. and Sambrook et al., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). (see in particular Screening By Hybridization 1.90 and Sequencing Denatured Double-Stranded DNA Templates 13.70). Illustrative of the invention, the polynucleotide set out in Table 1 [SEQ ID NO:1] was discovered in a DNA library derived from *Streptococcus pneumoniae* 0100993.

The DNA sequence set out in Table 1 [SEQ ID NOS:1] contains an open reading frame encoding a protein having about the number of amino acid residues set forth in Table 1 [SEQ ID NO:2] with a deduced molecular weight that can be calculated using amino acid

residue molecular weight values well known in the art. The polynucleotide of SEQ ID NO: 1, between nucleotide number 1 through number 1281 encodes the polypeptide of SEQ ID NO:2. The stop codon begins at nucleotide number 1284 of SEQ ID NO:1.11281

The aroA protein of the invention is structurally related to other proteins of the aro (5-enolpyruvylshikimate-3-phosphate synthase) family, as shown by the results of sequencing the DNA encoding aroA of the deposited strain. The protein exhibits greatest homology to Lactococcus lactis 5-enolpyruvoylshikimate -3-phosphate synthase (aro A) (showing a percent similarity of 79.7% and a percent identity of 68.2%) protein among known proteins. Other related sequences are:

Acc. No.	Species
X78413	*L.lactis* aroA
M80245	*B.subtilis* aroA
Z29339	*D.nodosus* aroA
I19982	Sequence 14 from patent US 5,512,466
L05004	*Staphylococcus aureus* aroA
X75325	*Synechocystis sp.* aroA
X77019	*P.pseudomallei* aro A
D90914	*Synechocystis sp.*aroA
X89371	*C.jejuni* aroA
L46372	*Yersinia pestis* aroA

Related sequences are described in Sharps. Analytical Biochem 140: 183-189, 1984; Majumder et al., Eur. J. Biochem 229: 99-106, 1995; O'Connell et al., J. Gen Microbiol. 139: 1449-1460, 1993; Oyston et al., "Immunization with live recombinant Salmonelle typhimurium aroA producing F1 antigen protects against plague," 1995; and Rogers et al., "Amplification of the aroA gene from Escherichia coli results in tolerance to the herbicide glyphosphate," 1983.

The invention provides a polynucleotide sequence identical over its entire length to the coding sequence in Table 1 [SEQ ID NO:1 or SEQ ID NO:2]. Also provided by the invention is the coding sequence for the mature polypeptide or a fragment thereof, by itself as well as the coding sequence for the mature polypeptide or a fragment in reading frame with other coding sequence, such as those encoding a leader or secretory sequence, a pre-, or pro- or prepro- protein sequence. The polynucleotide may also contain non-coding sequences, including for example, but not limited to non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns, polyadenylation signals, and additional coding sequence which encode additional amino acids. For example, a marker sequence that facilitates purification

of the fused polypeptide can be encoded. In certain embodiments of the invention, the marker sequence is a hexa-histidine peptide, as provided in the pQE vector (Qiagen, Inc.) and described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA 86:* 821-824 (1989), or an HA tag (Wilson *et al.*, *Cell 37:* 767 (1984). Polynucleotides of the invention also include, but are not limited to, polynucleotides comprising a structural gene and its naturally associated sequences that control gene expression.

A preferred embodiment of the invention is a polynucleotide of comprising nucleotide 1 to 1281 or 1284 set forth in SEQ ID NO:1 of Table 1 which encode the aroA polypeptide.

The invention also includes polynucleotides of the formula set forth in Table 1 (C)[SEQ ID NO:1] wherein, at the 5' end of the molecule, X is hydrogen, and at the 3' end of the molecule, Y is hydrogen or a metal, R_1 and R_2 is any nucleic acid residue, and n is an integer between 1 and 1000. Any stretch of nucleic acid residues denoted by either R group, where R is greater than 1, may be either a heteropolymer or a homopolymer, preferably a heteropolymer.

The term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides that include a sequence encoding a polypeptide of the invention, particularly a bacterial polypeptide and more particularly a polypeptide of the *Streptococcus pneumoniae* aroA having the amino acid sequence set out in Table 1 [SEQ ID NO:2]. The term also encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by integrated phage or an insertion sequence or editing) together with additional regions, that also may contain coding and/or non-coding sequences.

The invention further relates to variants of the polynucleotides described herein that encode for variants of the polypeptide having the deduced amino acid sequence of Table 1 [SEQ ID NO:2]. Variants that are fragments of the polynucleotides of the invention may be used to synthesize full-length polynucleotides of the invention.

Further particularly preferred embodiments are polynucleotides encoding aroA variants, that have the amino acid sequence of aroA polypeptide of Table 1 [SEQ ID NO:2] in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, that do not alter the properties and activities of aroA.

Further preferred embodiments of the invention are polynucleotides that are at least 70% identical over their entire length to a polynucleotide encoding aroA polypeptide having

an amino acid sequence set out in Table 1 [SEQ ID NOS:2 and 4], and polynucleotides that are complementary to such polynucleotides. Alternatively, most highly preferred are polynucleotides that comprise a region that is at least 80% identical over its entire length to a polynucleotide encoding aroA polypeptide of the deposited strain and polynucleotides complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length to the same are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

Preferred embodiments are polynucleotides that encode polypeptides that retain substantially the same biological function or activity as the mature polypeptide encoded by the DNA of Table 1 [SEQ ID NO:1].

The invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the invention especially relates to polynucleotides that hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the terms "stringent conditions" and "stringent hybridization conditions" mean hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. An example of stringent hybridization conditions is overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1x SSC at about 65°C. Hybridization and wash conditions are well known and exemplified in Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), particularly Chapter 11 therein.

The invention also provides a polynucleotide consisting essentially of a polynucleotide sequence obtainable by screening an appropriate library containing the complete gene for a polynucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 under stringent hybridization conditions with a probe having the sequence of said polynucleotide sequence set forth in SEQ ID NO:1 or a fragment thereof; and isolating said DNA sequence. Fragments useful for obtaining such a polynucleotide include, for example, probes and primers described elsewhere herein.

As discussed additionally herein regarding polynucleotide assays of the invention, for instance, polynucleotides of the invention as discussed above, may be used as a hybridization probe for RNA, cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding aroA and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the aroA gene. Such probes generally will comprise at least 15 bases. Preferably, such probes will have at least 30 bases and may have at least 50 bases. Particularly preferred probes will have at least 30 bases and will have 50 bases or less.

For example, the coding region of the aroA gene may be isolated by screening using the DNA sequence provided in SEQ ID NO: 1 to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the invention is then used to screen a library of cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polynucleotides and polypeptides of the invention may be employed, for example, as research reagents and materials for discovery of treatments of and diagnostics for disease, particularly human disease, as further discussed herein relating to polynucleotide assays.

Polynucleotides of the invention that are oligonucleotides derived from the sequences of SEQ ID NOS:1 and/or 2 may be used in the processes herein as described, but preferably for PCR, to determine whether or not the polynucleotides identified herein in whole or in part are transcribed in bacteria in infected tissue. It is recognized that such sequences will also have utility in diagnosis of the stage of infection and type of infection the pathogen has attained.

The invention also provides polynucleotides that may encode a polypeptide that is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, may allow protein transport, may lengthen or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in vivo*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. When prosequences are removed such inactive precursors generally are activated. Some or all of the prosequences may be removed before activation. Generally, such precursors are called proproteins.

In addition to the standard A, G, C, T/U representations for nucleotides, the term "N" may also be used in describing certain polynucleotides of the invention. "N" means that any of the four DNA or RNA nucleotides may appear at such a designated position in the DNA or RNA sequence, except it is preferred that N is not a nucleic acid that when taken in combination with adjacent nucleotide positions, when read in the correct reading frame, would have the effect of generating a premature termination codon in such reading frame.

In sum, a polynucleotide of the invention may encode a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences that are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

Vectors, host cells, expression

The invention also relates to vectors that comprise a polynucleotide or polynucleotides of the invention, host cells that are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the invention.

For recombinant production, host cells can be genetically engineered to incorporate expression systems or portions thereof or polynucleotides of the invention. Introduction of a polynucleotide into the host cell can be effected by methods described in many standard laboratory manuals, such as Davis et al., *BASIC METHODS IN MOLECULAR BIOLOGY*, (1986) and Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), such as, calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction and infection.

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, enterococci *E. coli*, streptomyces and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

A great variety of expression systems can be used to produce the polypeptides of the invention. Such vectors include, among others, chromosomal, episomal and virus-derived

vectors, *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression in this regard. The appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL*, (*supra*).

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

Polypeptides of the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Diagnostic Assays

This invention is also related to the use of the aroA polynucleotides of the invention for use as diagnostic reagents. Detection of aroA in a eukaryote, particularly a mammal, and especially a human, will provide a diagnostic method for diagnosis of a disease. Eukaryotes (herein also "individual(s)"), particularly mammals, and especially humans, infected with an organism comprising the aroA gene may be detected at the nucleic acid level by a variety of techniques.

Nucleic acids for diagnosis may be obtained from an infected individual's cells and tissues, such as bone, blood, muscle, cartilage, and skin. Genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR or other amplification technique prior to analysis. RNA or cDNA may also be used in the same ways. Using

amplification, characterization of the species and strain of prokaryote present in an individual, may be made by an analysis of the genotype of the prokaryote gene. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the genotype of a reference sequence. Point mutations can be identified by hybridizing amplified DNA to labeled aroA polynucleotide sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected by alterations in the electrophoretic mobility of the DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing. See, *e.g.*, Myers et al., *Science, 230:* 1242 (1985). Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or a chemical cleavage method. See, *e.g.*, Cotton et al., *Proc. Natl. Acad. Sci., USA, 85:* 4397-4401
(1985).

Cells carrying mutations or polymorphisms in the gene of the invention may also be detected at the DNA level by a variety of techniques, to allow for serotyping, for example. For example, RT-PCR can be used to detect mutations. It is particularly preferred to used RT-PCR in conjunction with automated detection systems, such as, for example, GeneScan. RNA or cDNA may also be used for the same purpose, PCR or RT-PCR. As an example, PCR primers complementary to a nucleic acid encoding aroA can be used to identify and analyze mutations.

The invention further provides these primers with 1, 2, 3 or 4 nucleotides removed from the 5' and/or the 3' end. These primers may be used for, among other things, amplifying aroA DNA isolated from a sample derived from an individual. The primers may be used to amplify the gene isolated from an infected individual such that the gene may then be subject to various techniques for elucidation of the DNA sequence. In this way, mutations in the DNA sequence may be detected and used to diagnose infection and to serotype and/or classify the infectious agent.

The invention further provides a process for diagnosing, disease, preferably bacterial infections, more preferably infections by *Streptococcus pneumoniae*, and most preferably otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid, comprising determining from a sample derived from an individual a increased level of expression of polynucleotide having the sequence of Table 1 [SEQ ID NO: 1]. Increased or decreased expression of aroA polynucleotide can be measured using

any on of the methods well known in the art for the quantation of polynucleotides, such as, for example, amplification, PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods.

In addition, a diagnostic assay in accordance with the invention for detecting over-expression of aroA protein compared to normal control tissue samples may be used to detect the presence of an infection, for example. Assay techniques that can be used to determine levels of a aroA protein, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Antibodies

The polypeptides of the invention or variants thereof, or cells expressing them can be used as an immunogen to produce antibodies immunospecific for such polypeptides. "Antibodies" as used herein includes monoclonal and polyclonal antibodies, chimeric, single chain, simianized antibodies and humanized antibodies, as well as Fab fragments, including the products of an Fab immunolglobulin expression library.

Antibodies generated against the polypeptides of the invention can be obtained by administering the polypeptides or epitope-bearing fragments, analogues or cells to an animal, preferably a nonhuman, using routine protocols. For preparation of monoclonal antibodies, any technique known in the art that provides antibodies produced by continuous cell line cultures can be used. Examples include various techniques, such as those in Kohler, G. and Milstein, C., *Nature 256:* 495-497 (1975); Kozbor *et al.*, *Immunology Today 4:* 72 (1983); Cole et al., pg. 77-96 in *MONOCLONAL ANTIBODIES AND CANCER THERAPY*, Alan R. Liss, Inc. (1985).

Techniques for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies.

Alternatively phage display technology may be utilized to select antibody genes with binding activities towards the polypeptide either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-aroA or from naive libraries (McCafferty, J. et al., (1990), Nature 348, 552-554; Marks, J. et al., (1992) Biotechnology *10*, 779-783). The affinity of these antibodies can also be improved by chain shuffling (Clackson, T. et al., (1991) Nature 352, 624-628).

If two antigen binding domains are present each domain may be directed against a different epitope - termed 'bispecific' antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptides to purify the polypeptides by affinity chromatography.

Thus, among others, antibodies against aroA- polypeptide may be employed to treat infections, particularly bacterial infections and especially otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid.

Polypeptide variants include antigenically, epitopically or immunologically equivalent variants that form a particular aspect of this invention. The term "antigenically equivalent derivative" as used herein encompasses a polypeptide or its equivalent which will be specifically recognized by certain antibodies which, when raised to the protein or polypeptide according to the invention, interfere with the immediate physical interaction between pathogen and mammalian host. The term "immunologically equivalent derivative" as used herein encompasses a peptide or its equivalent which when used in a suitable formulation to raise antibodies in a vertebrate, the antibodies act to interfere with the immediate physical interaction between pathogen and mammalian host.

The polypeptide, such as an antigenically or immunologically equivalent derivative or a fusion protein thereof is used as an antigen to immunize a mouse or other animal such as a rat or chicken. The fusion protein may provide stability to the polypeptide. The antigen may be associated, for example by conjugation, with an immunogenic carrier protein for example bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). Alternatively a multiple antigenic peptide comprising multiple copies of the protein or polypeptide, or an antigenically or immunologically equivalent polypeptide thereof may be sufficiently antigenic to improve immunogenicity so as to obviate the use of a carrier.

Preferably, the antibody or variant thereof is modified to make it less immunogenic in the individual. For example, if the individual is human the antibody may most preferably be "humanized"; where the complimentarity determining region(s) of the hybridoma-derived antibody has been transplanted into a human monoclonal antibody , for example as described in Jones, P. et al. (1986), *Nature* 321, 522-525 or Tempest et al., (1991) *Biotechnology* 9, 266-273.

The use of a polynucleotide of the invention in genetic immunization will preferably employ a suitable delivery method such as direct injection of plasmid DNA into muscles (Wolff et al., Hum Mol Genet 1992, 1:363, Manthorpe et al., Hum. Gene Ther.

1963:4, 419), delivery of DNA complexed with specific protein carriers (Wu et al., *J Biol Chem.* 1989: 264,16985), coprecipitation of DNA with calcium phosphate (Benvenisty & Reshef, *PNAS USA*, 1986:83,9551), encapsulation of DNA in various forms of liposomes (Kaneda et al., *Science* 1989:243,375), particle bombardment (Tang et al., *Nature* 1992, 356:152, Eisenbraun et al., *DNA Cell Biol* 1993, 12:791) and *in vivo* infection using cloned retroviral vectors (Seeger et al., *PNAS USA* 1984:81,5849).

Mechanisms of Action and Methods of Use

Polypeptides of the invention may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates and ligands may be natural substrates and ligands or may be structural or functional mimetics. See, *e.g.*, Coligan *et al.*, *Current Protocols in Immunology* 1(2): Chapter 5 (1991).

The invention also provides a method of screening compounds to identify those which enhance (agonist) or block (antagonist) the action of aroA polypeptides or polynucleotides, particularly those compounds that are bacteriostatic and/or bacteriocidal. The method of screening may involve high-throughput techniques. For example, to screen for agonists or antagoists, a synthetic reaction mix, a cellular compartment, such as a membrane, cell envelope or cell wall, or a preparation of any thereof, comprising aroA polypeptide and a labeled substrate or ligand of such polypeptide is incubated in the absence or the presence of a candidate molecule that may be a aroA agonist or antagonist. The ability of the candidate molecule to agonize or antagonize the aroA polypeptide is reflected in decreased binding of the labeled ligand or decreased production of product from such substrate. Molecules that bind gratuitously, *i.e.*, without inducing the effects of aroA polypeptide are most likely to be good antagonists. Molecules that bind well and increase the rate of product production from substrate are agonists. Detection of the rate or level of production of product from substrate may be enhanced by using a reporter system. Reporter systems that may be useful in this regard include but are not limited to colorimetric labeled substrate converted into product, a reporter gene that is responsive to changes in aroA polynucleotide or polypeptide activity, and binding assays known in the art.

Another example of an assay for aroA antagonists is a competitive assay that combines aroA and a potential antagonist with aroA-binding molecules, recombinant aroA binding molecules, natural substrates or ligands, or substrate or ligand mimetics, under appropriate conditions for a competitive inhibition assay. The aroA protein can be labeled, such as by radioactivity or a colorimetric compound, such that the number of aroA molecules

bound to a binding molecule or converted to product can be determined accurately to assess the effectiveness of the potential antagonist.

It is preferred that the agonists and antagonists of the invention act by altering the reversible transformation of phospho(enol)pyruvate (PEP) and shikimate 3-phosphate (S3P) to EPSP and inorganic phosphate (Pi).

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polynucleotide or polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a binding molecule, without inducing aroA-induced activities, thereby preventing the action of aroA by excluding aroA from binding.

Small organic molecules of the invention preferably have a molecular weight below 2,000 daltons, more preferably between 300 and 1,000 daltons, and most preferably between 400 and 700 daltons. An embodiment provides compounds that were not published prior to the filing date of this application. Another embodiment provides compounds excluding glyphosate (*N*-phosphonomethylglycine, GLP) and/or transition state inhibitors or transition state mimetics.

Potential antagonists include a small molecule that binds to and occupies the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or peptide-like molecules. Other potential antagonists include antisense molecules (see Okano, *J. Neurochem.* 56: 560 (1991); *OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION*, CRC Press, Boca Raton, FL (1988), for a description of these molecules). Preferred potential antagonists include compounds related to and variants of aroA.

Each of the DNA sequences provided herein may be used in the discovery and development of antibacterial compounds. The encoded protein, upon expression, can be used as a target for the screening of antibacterial drugs. Additionally, the DNA sequences encoding the amino terminal regions of the encoded protein or Shine-Delgarno or other translation facilitating sequences of the respective mRNA can be used to construct antisense sequences to control the expression of the coding sequence of interest.

The invention also provides the use of the polypeptide, polynucleotide or inhibitor of the invention to interfere with the initial physical interaction between a pathogen and mammalian host responsible for sequelae of infection. In particular the molecules of the

invention may be used: in the prevention of adhesion of bacteria, in particular gram positive bacteria, to mammalian extracellular matrix proteins on in-dwelling devices or to extracellular matrix proteins in wounds; to block aroA protein-mediated mammalian cell invasion by, for example, initiating phosphorylation of mammalian tyrosine kinases (Rosenshine *et al.*, *Infect. Immun.* 60:2211 (1992); to block bacterial adhesion between mammalian extracellular matrix proteins and bacterial aroA proteins that mediate tissue damage and; to block the normal progression of pathogenesis in infections initiated other than by the implantation of in-dwelling devices or by other surgical techniques.

The antagonists and agonists of the invention may be employed, for instance, to inhibit and treat otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid.

Vaccines

Another aspect of the invention relates to a method for inducing an immunological response in an individual, particularly a mammal which comprises inoculating the individual with aroA, or a fragment or variant thereof, adequate to produce antibody and/ or T cell immune response to protect said individual from infection, particularly bacterial infection and most particularly *Streptococcus pneumoniae* infection. Also provided are methods whereby such immunological response slows bacterial replication. Yet another aspect of the invention relates to a method of inducing immunological response in an individual which comprises delivering to such individual a nucleic acid vector to direct expression of aroA, or a fragment or a variant thereof, for expressing aroA, or a fragment or a variant thereof *in vivo* in order to induce an immunological response, such as, to produce antibody and/ or T cell immune response, including, for example, cytokine-producing T cells or cytotoxic T cells, to protect said individual from disease, whether that disease is already established within the individual or not. One way of administering the gene is by accelerating it into the desired cells as a coating on particles or otherwise.

Such nucleic acid vector may comprise DNA, RNA, a modified nucleic acid, or a DNA/RNA hybrid.

A further aspect of the invention relates to an immunological composition which, when introduced into an individual capable or having induced within it an immunological response, induces an immunological response in such individual to a aroA or protein coded therefrom, wherein the composition comprises a recombinant aroA or protein coded therefrom comprising DNA which codes for and expresses an antigen of said aroA or

protein coded therefrom. The immunological response may be used therapeutically or prophylactically and may take the form of antibody immunity or cellular immunity such as that arising from CTL or CD4+ T cells.

A aroA polypeptide or a fragment thereof may be fused with co-protein which may not by itself produce antibodies, but is capable of stabilizing the first protein and producing a fused protein which will have immunogenic and protective properties. Thus fused recombinant protein, preferably further comprises an antigenic co-protein, such as lipoprotein D from *Hemophilus influenzae*, Glutathione-S-transferase (GST) or beta-galactosidase, relatively large co-proteins which solubilize the protein and facilitate production and purification thereof. Moreover, the co-protein may act as an adjuvant in the sense of providing a generalized stimulation of the immune system. The co-protein may be attached to either the amino or carboxy terminus of the first protein.

Provided by this invention are compositions, particularly vaccine compositions, and methods comprising the polypeptides or polynucleotides of the invention and immunostimulatory DNA sequences, such as those described in Sato, Y. *et al*. Science 273: 352 (1996).

Also, provided by this invention are methods using the described polynucleotide or particular fragments thereof which have been shown to encode non-variable regions of bacterial cell surface proteins in DNA constructs used in such genetic immunization experiments in animal models of infection with *Streptococcus pneumoniae* will be particularly useful for identifying protein epitopes able to provoke a prophylactic or therapeutic immune response. It is believed that this approach will allow for the subsequent preparation of monoclonal antibodies of particular value from the requisite organ of the animal successfully resisting or clearing infection for the development of prophylactic agents or therapeutic treatments of bacterial infection, particularly *Streptococcus pneumoniae* infection, in mammals, particularly humans.

The polypeptide may be used as an antigen for vaccination of a host to produce specific antibodies which protect against invasion of bacteria, for example by blocking adherence of bacteria to damaged tissue. Examples of tissue damage include wounds in skin or connective tissue caused, e.g., by mechanical, chemical or thermal damage or by implantation of indwelling devices, or wounds in the mucous membranes, such as the mouth, mammary glands, urethra or vagina.

The invention also includes a vaccine formulation which comprises an immunogenic recombinant protein of the invention together with a suitable carrier. Since

the protein may be broken down in the stomach, it is preferably administered parenterally, including, for example, administration that is subcutaneous, intramuscular, intravenous, or intradermal. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation insotonic with the bodily fluid, preferably the blood, of the individual; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

While the invention has been described with reference to certain aroA protein, it is to be understood that this covers fragments of the naturally occurring protein and similar proteins with additions, deletions or substitutions which do not substantially affect the immunogenic properties of the recombinant protein.

Compositions, kits and administration

The invention also relates to compositions comprising the polynucleotide or the polypeptides discussed above or their agonists or antagonists. The polypeptides of the invention may be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of a polypeptide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. The formulation should suit the mode of administration. The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention.

Polypeptides and other compounds of the invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 mg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

In-dwelling devices include surgical implants, prosthetic devices and catheters, i.e., devices that are introduced to the body of an individual and remain in position for an extended time. Such devices include, for example, artificial joints, heart valves, pacemakers, vascular grafts, vascular catheters, cerebrospinal fluid shunts, urinary catheters, continuous ambulatory peritoneal dialysis (CAPD) catheters.

The composition of the invention may be administered by injection to achieve a systemic effect against relevant bacteria shortly before insertion of an in-dwelling device. Treatment may be continued after surgery during the in-body time of the device. In addition, the composition could also be used to broaden perioperative cover for any surgical technique to prevent bacterial wound infections, especially *Streptococcus pneumoniae* wound infections.

Many orthopaedic surgeons consider that humans with prosthetic joints should be considered for antibiotic prophylaxis before dental treatment that could produce a bacteremia. Late deep infection is a serious complication sometimes leading to loss of the prosthetic joint and is accompanied by significant morbidity and mortality. It may therefore

be possible to extend the use of the active agent as a replacement for prophylactic antibiotics in this situation.

In addition to the therapy described above, the compositions of this invention may be used generally as a wound treatment agent to prevent adhesion of bacteria to matrix proteins exposed in wound tissue and for prophylactic use in dental treatment as an alternative to, or in conjunction with, antibiotic prophylaxis.

Alternatively, the composition of the invention may be used to bathe an indwelling device immediately before insertion. The active agent will preferably be present at a concentration of 1µg/ml to 10mg/ml for bathing of wounds or indwelling devices.

A vaccine composition is conveniently in injectable form. Conventional adjuvants may be employed to enhance the immune response. A suitable unit dose for vaccination is 0.5-5 microgram/kg of antigen, and such dose is preferably administered 1-3 times and with an interval of 1-3 weeks. With the indicated dose range, no adverse toxicological effects will be observed with the compounds of the invention which would preclude their administration to suitable individuals.

Each reference disclosed herein is incorporated by reference herein in its entirety. Any patent application to which this application claims priority is also incorporated by reference herein in its entirety.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1 Strain selection, Library Production and Sequencing

The polynucleotide having the DNA sequence given in SEQ ID NO:1 was obtained from a library of clones of chromosomal DNA of *Streptococcus pneumoniae* in *E. coli*. The sequencing data from two or more clones containing overlapping *Streptococcus pneumoniae* DNAs was used to construct the contiguous DNA sequence in SEQ ID NO:1. Libraries may be prepared by routine methods, for example:

Methods 1 and 2 below.

Total cellular DNA is isolated from *Streptococcus pneumoniae* 0100993 according to standard procedures and size-fractionated by either of two methods.

Method 1

Total cellular DNA is mechanically sheared by passage through a needle in order to size-fractionate according to standard procedures. DNA fragments of up to 11kbp in size are rendered blunt by treatment with exonuclease and DNA polymerase, and EcoRI linkers added. Fragments are ligated into the vector Lambda ZapII that has been cut with EcoRI, the library packaged by standard procedures and *E.coli* infected with the packaged library. The library is amplified by standard procedures.

Method 2

Total cellular DNA is partially hydrolyzed with a one or a combination of restriction enzymes appropriate to generate a series of fragments for cloning into library vectors (e.g., RsaI, PalI, AluI, Bshl235I), and such fragments are size-fractionated according to standard procedures. EcoRI linkers are ligated to the DNA and the fragments then ligated into the vector Lambda ZapII that have been cut with EcoRI, the library packaged by standard procedures, and *E.coli* infected with the packaged library. The library is amplified by standard procedures.

Example 2 aroA Characterization: Steady-State Kinetic Studies of 5-Enolpyruvylshikimate-3-phosphate Synthase from *Streptococcus pneumoniae*

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) is the key enzyme in the aromatic amino acid biosynthesis pathway (the "shikimate pathway") (1). The enzyme catalyzes an unusual reversible transformation of phospho(enol)pyruvate (PEP) and shikimate 3-phosphate (S3P) to EPSP and inorganic phosphate (Pi) (**Scheme 1**). Intense interest in this enzyme has been provoked by the discovery that the broad-spectrum, post-emergence herbicide glyphosate (*N*-phosphonomethylglycine, GLP) inhibits this single enzyme (2) by functioning as a transition-state inhibitor (3) (**Scheme 2**).

Provided herein is the identification of EPSP synthase in *Streptococcus pneumoniae*, and purification of the enzyme to homogeneity (4). Further provided herein are results of a comprehensive steady-state kinetic study of this enzyme, aimed at deducing its kinetic mechanism, especially the order of substrate binding in both forward and reverse reactions. The inhibition patterns of the enzyme by GLP and respective enzyme reaction product are described.

Since the EPSP synthase reaction is freely reversible, the enzyme can be assayed in both forward and reverse directions. In the forward reaction, the production of Pi is monitored by fluorescence change when coupled to the nucleoside phosphorylase and 7-methylguanosine reaction (5); in the reverse reaction, PEP is coupled to pyruvate kinase

and lactate dehydrogenase and the change in absorbance at 340 nm (NADH) was detected (6).

In the forward reaction, GLP is a competitive inhibitor with respect to PEP, but an uncompetitive inhibitor relative to S3P (**Figure 1**).

Product inhibition in the forward reaction by EPSP shows that EPSP is a competitive inhibitor versus both PEP and S3P (**Figure 2**).

In the reverse reaction, GLP led to uncompetitive inhibition versus EPSP, but noncompetitive inhibition versus Pi (**Figure 3**).

Product inhibition in the reverse reaction by S3P shows that S3P competitively inhibits the enzyme versus EPSP, but appears to be an uncompetitive inhibitor versus Pi (**Figure 4**).

The patterns of inhibition of *S. pneumoniae* EPSP synthase and the corresponding kinetic constants are summarized in **Table I**.

Based on these results, we propose a random sequential mechanism for the forward reaction of *S. pneumoniae* EPSP synthase (**Scheme 3**); but the current data are not sufficient to pinpoint the mechanism in the reverse reaction.

The herbicide glyphosate is an inhibitor of *S. pneumoniae* EPSP synthase. The inhibition of EPSP synthase by glyphosate in both forward and reverse reactions has been characterized by steady-state kinetics.

Product inhibition of the enzyme in both directions has also been characterized. The results support a random sequential mechanism in the forward reaction, but deducing the mechanism in the reverse reaction requires further investigation.

A number of compounds were screened against EPSP synthase *in vitro* and were shown to comparably inhibit the enzyme. MICs against *S.pneumoniae* and *E.coli* also demostarted that these compounds were active antibacterial compounds. Analysis of these compounds indicates they are active site directed and one compound has been shown to be competitive with the shikimate-3-phosphate substrate.

Table I. Inhibition patterns and kinetic constants of *S. pneumoniae* EPSP synthase.

Direction of Reaction	Inhibitor versus Substrate	Mode of Inhibition[a]	K_i, microMolar
Forward	GLP vs. PEP	Competitive	2.8±0.3

	GLP vs. S3P	Uncompetitive	65.6±2.7
	EPSP vs. PEP	Competitive	329±26
	EPSP vs. S3P	Competitive	44.0±2.4
Reverse	GLP vs. EPSP	Uncompetitive	625±23
	GLP vs. Pi	Noncompetitive	1136±60
	S3P vs. EPSP	Competitive	12.8±2.1
	S3P vs. Pi	Uncompetitive	4936±158

[a] The mode of inhibition was determined by the associated error and the reduced Chi^2 of fitting the data into equations in GraFit (*v*4.06, Erithacus Software Ltd.). The inhibition patterns represent the best fit of the data to the respective models.

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